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Characterization of Peroxidase Enzyme from Water Spinach (*Ipomoea aquatica* Forssk.) Fraction

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Abstract

Peroxide enzymes are widely used as a reagent in many clinical diagnosis of analysis methods such as ELISA, immunoblotting, and immunochemistry. Water spinach (*Ipomea aquatica* Forssk) is a species of plant available abundantly in Indonesia, hence it is a potential source of peroxidase. In this study, the crude extract of water spinach leaves was known to contain peroxidase enzyme which was active toward hydrogen peroxide. A fraction of extract yielded from differential fractionation using ammonium sulfate at concentration 40-65% (F4) was known to be the most active. Characterization of specific activity of peroxidase of F4 for optimum pH and temperature was at pH 5 and 60°C. The Michaelis-Menten constant (Km) was 0,27 mg/mL and enzyme's catalytic rate (Vmax) was 2,16 mg/mL/min.

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*Keywords:*peroxidase; water spinach leaves; enzyme activity

1. Introduction

Enzymes are the functional units of cell metabolism. Working in a fixed sequence, enzyme catalyzes many reactions such as the disintegration of nutrient molecules and reactions that deposit and transform chemical energy. Through its activities, a well-coordinated enzyme system produces a harmonious relationship between different metabolic activities, which is necessary to sustain life[1].

Peroxidase is a member of oxidoreductase enzymes that catalyze wide varieties of oxidation-reduction reactions. The enzyme peroxidase is an enzyme group that is quite important, its use is very broad and includes a variety of

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uses, including in the field of environment, the synthesis of resins, compounds biotransformation diagnostics, and pharmaceuticals[2]. Peroxidase is one of the enzymes used in biochemical analysis, as reagents in clinical diagnostics and in the enzyme immunoassay. Peroxidase is also used to examine metabolites by catalyzing oxidation of hydrogen peroxide which is usually generated from other enzymatic reactions related to metabolites in the body that needs to be measured[3, 4].

Peroxidase enzymes have been found in various species of microorganisms and plants, such as latex, figs, bananas, apples, potatoes, carrots and legumes[5]. While the peroxidase enzyme from kiwi, tomatoes and horseradish has been successfully purified. Even though peroxidase is found in many plants, but to date the source of commercially available peroxidase is derived from horseradish roots extraction (*Armoracia rusticana*) widely grown in sub-tropical countries[6]. Peroxidase of *Ipomoea* is a good source of peroxide as an alternative to Horseradish Peroxidase (HRP)[7]. One of the plants in the genus *Ipomoea* is *Ipomoea aquatica* Forssk (water spinach). According to Gusyana[8], water spinach contains high peroxidase activity due to the iron content in spinach that functions as a coenzyme.

So far, water spinach is a vegetable that is generally treated as a comestible plant. Water spinach is easy to find because it is commonly cultivated in the Southeast Asian region, including Indonesia[9]. Therefore, the use of water spinach as a source of peroxidase enzyme is an alternative to retrieve more potential peroxidase enzyme and also cheaper source. According to a research conducted by Sthadini[10], water spinach crude extract containing peroxidase with a relatively high activity was at the optimum temperature of 50° C and pH 6.

This study is a continuation of the previous study, aiming to characterize the optimum temperature and pH of peroxidase of water spinach leaves fraction. Characterization was also performed to determine the Michaelis Menten constant (Km), maximum catalytic velocity (Vmax) and molecular weight of peroxidase enzymes derived from *I. aquatica* Forssk fraction.

Nomenclature

ELISA	Enzyme Linked Immunosorbent Assay
Km	Michelis-Menten constant
Vmax	maximum catalytic velocity
HRP	Horseradish Peroxidase
BSA	Bovine Serum Albumin
SDS PAGE	Sodium Dodecyl Sulphonate

2. Experiments

2.1. Material

Materials: Water spinach leaves (*Ipomoea aquatica* Forssk.), obtained from plantation in Bandung Lembang area. Purified water was obtained using Lab Tech Automatic Water Still LWD 3008 and chemical agents were obtained from Merck and Sigma.

Instruments: Analytic scale (Mettler Toledo, AL 204), blender (Miyako, BI-101PL), pH-meter (Mettler Toledo, pH/Ion S220), spectrophotometer Uv-Vis (Shimadzu, UVmini-1240), centrifuge (Beckman J2-21), vortex mixer (IKA, Genius-3), micropippete (Boeco, Germany), electrophoresis apparatus (Bio-Rad, CA 94547).

2.2. Methods

2.2.1. Fractionation of Sample

The samples used in the research were water spinach leaves fraction (*Ipomoea aquatica* Forssk.). Fifty grams of washed water spinach and 200 mL of cool aquadest (±4ºC) were blended to create a crude extract. The crude extract was then centrifuged at 4000 rpm and 4ºC. The filtrates were fractionated differentially using ammonium sulfate at

concentrations of 0-10%, 10-20%, 20-40% and 40-65%[11]. We gained four fractions from this process, fraction 1 (F1), fraction 2 (F2), fraction 3 (F3) and fraction 4 (F4). Each fractionwas dialyzed to remove ammonium sulfate using cellophane bags. Dialysis was conducted for 48 hour at ± 4ºC.

2.2.2. Analysis

Activity of peroxidase in each fraction was measured at temperature of 50°C and pH6, referring to the optimum reaction temperature and pH of the crude extract ofwater spinach leaves and using the Trinder method to discover the most active fraction[10]. The principle of Trinder method is by using H_2O_2 as acceptor that reacts with4-aminoantipirin-phenol as the substrate which will produce red quinonimine. The quinonimine formed was measured at a wavelength of510nm. The protein level in each fraction was determined by Lowry method using *Bovine Serum Albumin* (BSA) as the standard.

2.2.3. Enzyme Characterization

Enzyme fractions with the highest activities were then characterized to determine the optimum temperature and pH on the specific activity of the enzyme. This was carried out simultaneously at various temperature, i.e. at room temperature, 40^o, 50^o, 60^o, and70^oC; and pH variations, namely pH5, 6, 7, 8, and 9. Additionally, there was also the determination of peroxidase enzyme's Michaelis Menten constant (Km) and maximum catalytic velocity (Vmax) value.SDS-PAGE method used to determine the level of purity and molecular weight of water spinach (*Ipomoea aquatica* Forssk) peroxidase.

3. Results and Discussion

Peroxidases in plant commonly found in the cell walls, vacuoles, cell organelle transport, and also on the rough endoplasmic reticulum[7]. On the process of extraction, water spinach leaves were first chopped in order to ease the process of homogenizing using a blender. Peroxidases inside the cell were released and dispersed in the water. Extraction was carried out at a temperature of 4^o C, referring to the stable temperature of horseradish peroxidase (HRP)[12].

The water spinach leaves extracts were then differentially fractionated using ammonium sulfate at concentrations of 0-10%, 10-20%, 20-40% and 40-65%, referring to white radish fractionation[11]. Protein can be purified by differential saturation of ammonium sulfate. Different concentrations of ammonium sulfate could precipitate different types of protein, thus this process could be used for the first step of protein purification.

Peroxidase enzyme activity assay in the fraction was performed using the Trinder method. This method uses the reduction-oxidation reaction principle of hydrogen peroxide and 4-aminoantipirin-phenol, catalyzed by peroxidase enzymes, which will yield the colored compound quinonimine. Absorbance of quinonimine formed was measured at a wavelength of 510 nm. Quinonimine absorption increases in proportion to the activity of the enzyme peroxidase. In this research, the peroxidase enzyme activity of extracts and fractions test were performed at a temperature of 50^oC and pH 6, referring to the research by Sthadini[10] where crude extracts of water spinach described to had the highest activity at these temperature and pH. The absorption measurement results of fraction and crude extracts of the water spinach leaves are listed in Table 1.It showed that fraction 4 (F4) produced the highest absorption value, compared to the other fractions. Thus it can be deduced that this fraction is the most active fraction.

Table 1. Absorbance & Peroxidase Activities of Water Spinach Leaves Crude Extract and Fraction

Samples	ΔAbsorbance (t0-t5/5)	Peroxidase Activity (Unit/mL enzyme)
Crude extract	0,0583	2,66
F1	0,0363	1,66
F2	0,0798	3,64
F3	0,0459	2,09
F4	0,1613	7,35

Concentrations of protein in the samples are presented in table 2. F4 contains smaller amount of protein than the crude extract of water spinach leaves, but had the highest activity of peroxidase. This indicates that the protein contained in F4 is mostly the peroxidase enzyme.

Table 2. Absorbance & Protein Concentrations of Water Spinach Leaves Crude Extract and Fraction

Sample	Δ Absorbance	Protein Concentrations (mg/mL)
Crude Extract	0,7850	0,062
F1	0,3767	0,022
F2	0,6847	0,052
F3	0,5782	0,042
F4	0,6911	0,053

F4 generated the highest activity in peroxidase activity (7.35 units/mL of enzyme) even though smaller in protein levels (0,053 mg/mL) than the water spinach's crude extract (0,062 mg/mL). This is possibly due to the crude extract that contains phenolic compounds which reacted with peroxidase during storage, thus decreasing the number of active enzymes.

Decline in activity in F1 indicated that proteins in the form of peroxidase enzymes were precipitated in small amounts, resulting in low enzyme activity value and small concentration of enzyme protein. Statistical analysis of theses results with one-way variance analysis (One-way ANOVA), with a confidence level of 95%, indicated that peroxidase enzyme activities in crude extracts and fractions of water spinach (F1-F4) differs significantly. Statistical analysis was then continued using Real Difference Test, with real level of 1%, indicated that the F4 sample had the highest average and significantly different compared to other samples of crude extract and fractions

The enzyme specific activity is a comparison between unit activity and protein levels. Specific activity values obtained from crude extract and F1- F4 fractions are presented in table 3. F4 has the highest specific activity at 147 units/mg of protein, with a degree of purity of 3.32 times higher than the crude extract and the other three enzyme fractions.

Table 3. Specific Activities of Water Spinach Leaves Crude Extract and Fractions

Sample	Specific Activity (Unit/mg protein)	Purity
Crude Extract	44,33	1
F1	83	1,87
F2	72,8	1,64
F3	52,25	1,18
F4	147	3,32

Electrophoresis was carried out to characterize the purity fraction resulting from dialysis and to determine the molecular weights. Coomassie blue R-250 was used in the staining process to determine the bands protein's movement positions. This was carried out to spot molecule samples that has been successfully separated. The purity of the peroxidase enzyme and molecular weight determinations made by SDS-PAGE can be seen in Fig. 1.

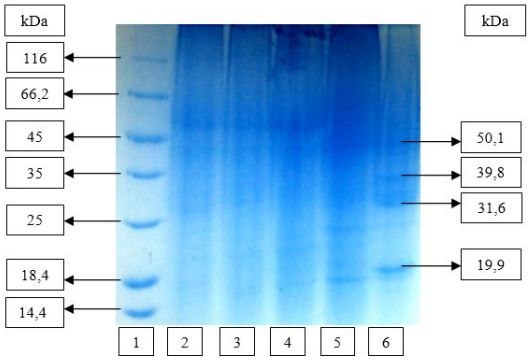

Fig. 1. Electroforegram of SDS-PAGE electrophoresis; 1: protein marker ; 2: crude extract; 3: F1; 4: F2; 5: F3; 6: F4.

F1, F2, F3, and F4 were still impure because they generated too much bands. But F4 had the highest purity among the fractionswhich was characterized by the emergence of some well separated bands of electrophoresis gel. It has molecular weights of 50.1; 39.8; 31.6; and 19.9 kDa.

The band that appeared on the molecular weight of 19.9 kDa was estimated to be water spinach's peroxidase enzyme. It has a molecular weight nearest to the molecular weight of the peroxidase enzyme on *Ipomoea palmata* of 20.1 kDa, purified using three-phase partitioning[7]. However, to obtain a better result, the F4 fraction still had to undergo the next stage of purification in order to obtain the pure peroxidase enzyme.

Enzyme activity can be affected by several factors, including the concentration of enzyme and substrate, pH, and temperature[13]. Peroxidase enzymes are produced from different sources and have different characteristics. Hence, peroxydase enzymes are characterized based on optimum temperature and pH determination, to gain the enzymes' optimum activity state.

Determination of the peroxidase enzyme activity was conducted on the most active fraction, which is F4, using the aforemantioned Trinder method. The determination of optimum pH and temperature was carried out simultaneously using pH variations (5, 6, 7, 8, 9) on 0.2 M phosphate solution and incubation temperature variations (room temperature [24^o], 40°, 50^o, 60^o, 70^{o}C). The acquired activity values of F4 shown on table 4.

Table 4: Water Spinach Peroxidase Activity at Different Temperature and pH

Temperature	Peroxidase Activity(Unit/mL enzyme)				
	pH 5	pH 6	pH 7	pH 8	pH 9
24^{o}C	1,89	2,24	1,93	2,06	1,07
40 ^{o}C	2,90	3,65	4,02	2,79	2,39
50 ^{o}C	9,75	7,69	5,12	3,57	1,45
60 ^{o}C	10,14	8,04	3,16	2,45	1,36
70 ^{o}C	7,24	6,93	3,06	2,32	1,11

Statistical analysis of the results using a simple factorial variant analysis (Two Way ANOVA) with a 95% confidence level indicated that there was an interaction between temperature and pH, which meant both of these factors (temperature and pH) had significant mutual influence on different peroxidase enzyme activities. Therefore,

a subsequent observation using a curve influence graphic of enzyme activity and temperature interaction was used to determine the optimum pH and temperature. (Fig. 2.).

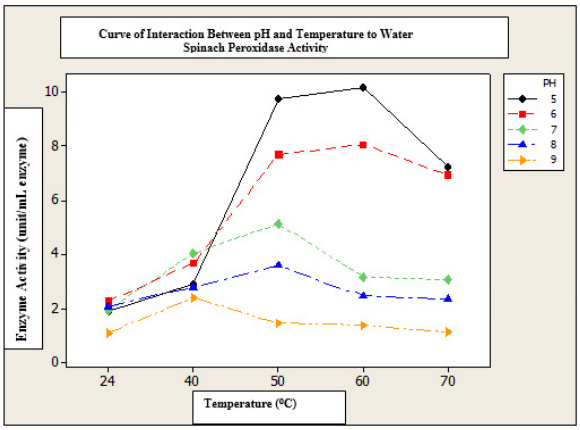

Fig. 2. Curve of interaction between pH and temperature to water spinach peroxidase.

Fig. 2. shows that the average of the highest peroxidase enzyme activity occured at 60°C (optimum temperature) and at pH 5 (optimum pH). Greater values than the optimum pH and temperature, a decrease in enzyme activity was observed. If pH and temperature values were above the optimum condition, the enzyme would undergo denaturation process. This would impair the active enzyme and thus reducing its effective concentrations, reaction speed, and ultimately lowering its activity[13].

The main character specified in enzyme's kinetic property is its maximum catalytic velocity (Vmax). This value is obtained from the increase in substrate's concentration value in oppose to the enzyme's concentration. The substrate's concentration when catalytic speed reaches half maximum is expressed as Michaelis-Menten constant (Km)[13].

The value of the catalytic velocity (V) is determined by measuring peroxidase enzyme activity from F4 at optimum pH and temperature (60°C pH 5) using different concentrations of hydrogen peroxide (0.004%, 0.006%, 0.008%, 0.01%, 0.012%, 0.014% and 0.016% b/v) as substrate [S]. The Km value for the water spinach leaves fraction peroxidase enzyme is 0.27 mg/mL and Vmax amounting to 2.16 mg/mL. Based on the obtained values of Km and Vmax, the concentration of the substrate used greatly affect enzyme activities.

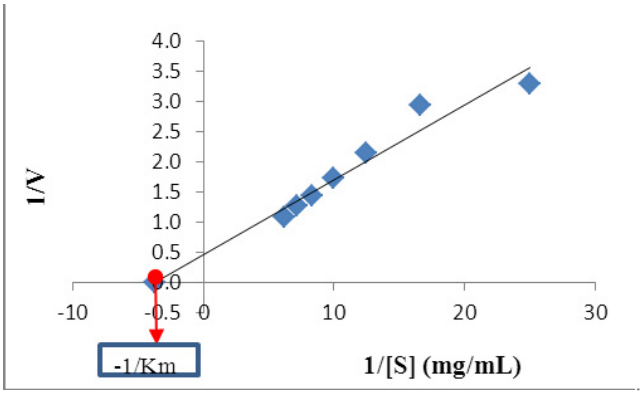

Fig. 3. Lineweaver-Burk curve (The relationship between 1/[S] with 1/V).

4. Conclusion

Peroxidase from water spinach leaves crude extract showed that it had activity toward hydrogen peroxide substrate. Fraction 4 (F4) which was yielded from differential fractionation using ammonium sulfate 40-65% was found to have the highest activity. Optimum pH and temperature of water spinach fraction peroxidase were at pH 5 and 60^{o}C. Meanwhile, the value of Michaelis-Menten constant (Km) and maximum catalytic velocity (Vmax) of the enzyme were 0.27 mg/mL and 2.16 mg/mL/ minute respectively.

Further purification of F4 fraction from water spinach leaves is needed in order to gain pure peroxidase enzymes, thus allowing subsequent research on molecular identification and characterization.

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